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CHROMATOGRAPHIC IMMUNOASSAY AND RELEASE TAGS(U)
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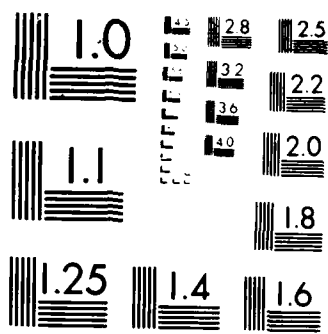
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19 ABSTRACT (Continue on reverse if necessary and identify by block number) Substrate-leash amplification has been demonstrated with ribonuclease fragments. A stable affinity column has been developed for purifying anti T-2 monoclonal antibody. A repetitive immunoassay device has been developed that detects as little as 1 ng of T-2 by using a Fab' fluorescein conjugate. Isomeric olefin release tags have been developed.				
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Annual Progress Report-3 for Contract N00014-84-C-0254
"Chromatographic Immunoassay and Release Tags"

(1) Project goals

→ The overall goal of ^{this} our project is to improve immunoassays in terms of their reliability, convenience, sensitivity and miniaturization for in-the-field testing. The work is also relevant to DNA probe assays. More specific goals are to:

- a develop ribonuclease (RNase) as an enzyme label for immunoassay.
- b increase the sensitivity for detecting RNase by introducing "substrate leash amplification"
- c develop affinity-separation immunoassay, a particularly rugged and convenient approach
- d determine T-2 toxin by an immunoassay
- e advance electrophoric release tags, a novel class of molecular labels that can be detected with high sensitivity and multiplicity
- f advance the usefulness of avidin-biotin as an accessory molecular binding tool for immunoassays

(2) Most recent accomplishments

- a Substrate-leash amplification, a new concept for chemical amplification, has been introduced. The system comprises fragments of RNase immobilized onto a chromatographic surface via polycytidylic acid as a substrate leash. More RNase activity elutes from a substrate-leash column than is applied. See manuscripts 1 and 2 in Section 4a below.
- b The problem of not having a stable leash for T-2 toxin has been overcome. This was done by activating T-2 with tresyl chloride followed by reaction onto an amino or hydrazide site. For example, T-2 was immobilized in this manner on hydrazide gel. Utilized as an affinity chromatography column, this T-2 gel purifies a monoclonal antibody for T-2 in high yield in a single step. See manuscript 3 and patent application 5.
- c The above antibody for T-2 toxin was converted to a Fab'fluorescein conjugate, and this was complexed onto the T-2 gel. This device, fabricated as a column, repetitively detects T-2 toxin. For example, 15 successive doses of T-2 ranging from 1 to 50 nanograms are detected using a column packed with a small volume (0.2 mL) of this gel without re-charging with Fab'fluorescein. This is the first example of such a repetitive immunoassay device. See manuscript 4 and patent application 6.

- d A pair of olefin release tags were prepared, starting from o- and m-hydroxyacetophenone. After an alkylation reaction with pentafluorobenzyl bromide, a Wittig reaction with methyl(triphenylphosphoranylidene) acetate, alkaline hydrolysis, and activation to an acid chloride with thionyl chloride, two isomeric release tags were obtained. They react to form a stable conjugate with a hydroxylic target compound, and the resulting conjugates can be cleaved by aqueous permanganate/periodate at the low picogram level with subsequent detection of the released electrophores by GC-ECD.

(3) Plans for next year

We will continue to optimize substrate-leash amplification, addressing the problems of RNase contamination and background leakage. In parallel, we will explore the use of RNase as an enzyme label in our hit-and-run immunoassay. It is attractive to employ a chain-cutting enzyme for this purpose not only for the enhanced sensitivity provided by substrate-leash amplification, but such an enzyme can release any type of secondary signal group, e.g. many dye or fluorescein molecules, from a surface. This gives flexibility and additional amplification for the final detection step. We will also continue our development of electrophoric release tags, and prepare biotin derivatives with enhanced binding to avidin relative to those available currently.

(4) a Publications and patents

1. Ehrat, M., Cecchini, D., and Giese, R.W. "Substrate-Leash Amplification with Ribonuclease S-Peptide and S-Protein", Clin Chem, 1986, **32**, 1622-1630.
2. Cecchini, D.J., Ehrat, M., and Giese, R.G. "Substrate-Leash Amplification of Ribonuclease Activity", J. Am. Chem. Soc., in press.
3. Allam, K.I., Ehrat, M., Cecchini, D., Warden, B.A., and Giese, R.W. "Tresyl Activation of A Hydroxyalkyl Ligand for Coupling to a Hydrazide Gel: Stable Immobilization of T-2 Toxin for Affinity Purification of T-2 Antibody", Anal. Biochem., in press.
4. Warden, B.A., Allam, K., Sentissi, A., Cecchini, D.J., and Giese, R.W. "Repetitive Hit-and-Run Fluoroimmunoassay for T-2 Toxin", submitted for publication.
5. Giese, R.W., Warden, B.A., Allam, K.I., Ehrat, M., Cecchini, D.J., and Sentissi, A. "Repetitive Hit-And-Run Immunoassay and Stable Support Analyte Conjugates; Applied to T-2 Toxin", patent application submitted.

6. Giese, R.W., Ehrat, M., and Cecchini, D. "An Enzyme Amplification Method and Compositions For Its Implementation", patent application submitted.

b Awards and honors

None

c Major presentation

Three presentations on substrate-leash amplification

- i Army Research Office Fourth Biodection Workshop, April 16, 1986, Cashiers, NC
- ii Oak Ridge Conference on Advanced Concepts for the Clinical Laboratory, April 17, 1986, Knoxville, TN
- iii Tenth International Symposium on Column Liquid Chromatography, May 22, 1986, San Francisco, CA

One presentation on release tags

- i American Chemical Society National Meeting, April 13, 1986, New York. The presentation was selected as newsworthy by Chemical and Engineering News (April 28, 1986, p. 68).

d Graduate students

Russell Garlick, Caucasian

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Substrate-Leash Amplification with Ribonuclease S-Peptide and S-Protein

Markus Ehrat,¹ Douglas J. Cecchini, and Roger W. Giese²

The S-peptide and S-protein fragments of ribonuclease S (RNase S, no EC no. assigned) have been immobilized onto separate Sepharose gels via a "leash" of polycytidylic acid substrate. Each of these gels releases its RNase fragment when treated with the complementary enzyme fragment or with RNase A (EC 3.1.27.5), and the released fragments recombine to give RNase S activity. Thus this system provides substrate-leash amplification (SLA), such that more enzymatic activity is eluted from the system than is applied. For example, 100 pg of RNase applied to the S-peptide gel is amplified by 1.9×10^4 to the equivalent of 1.9 μ g of activity in 20 h, when followed by combination of the released S-peptide with excess S-protein. We also tested a three-stage amplification system, with a pair of S-peptide and S-protein gels at each stage. In this system the cumulative amplification of the initial 1-ng dose of RNase A is 4.9, 52, and 25-fold after each stage, respectively. Only 2 mg of each SLA gel is used per stage in these experiments, reflecting the magnitude of their production of RNase S activity.

Additional Keyphrases: enzyme activity · signal amplification · peptide and protein fragments immobilized on agarose gel · polycytidylic acid · ribonuclease A

Chemical amplification, the formation of an enhanced chemical response, can occur in three ways. A common example of the first, *catalytic amplification*, is the action of an enzyme or coenzyme; substrate cycling (1), in which a substrate acts as a catalyst first by participating in one reaction, then cycling back to its original state in a second reaction, also illustrates this process. In *gate amplification*, the opening of a molecular gate, such as a channel in a membrane, amplifies the passage of molecules from one zone to another. Complement, for example, has such an effect on a target cell. In *multiplicative amplification*, the amount of a substance is multiplied by a constant factor repetitively, e.g., the unhindered growth of successive generations of a virus. These three mechanisms of amplification also can occur in combination. For example, in the overall action of immune complement, catalysis and gate mechanisms are both present.

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The role of chemical amplification in chemical analysis, including clinical chemistry, was carefully reviewed in 1978 by Blaedel and Boguslaski (2), who discussed such amplification components as enzymes, coenzymes, inorganic iodide, catalytic electrodes, bacteriophage, and liposomes. When use of such components does not inherently provide the specificity required, or when they do not interact directly with the analyte, then an antibody, secondary enzyme, or secondary chemical reaction is used to make the amplification system specific or analyte-responsive. Aside from the simple use of enzymes as amplification catalysts, many of the techniques discussed by those authors were not very practical for clinical analysis at that time, the most general problem being the requirement of these techniques for complex, highly purified reagents, with the accompanying secondary difficulties of short shelf-life and high cost.

A recent example of gate amplification in chemical analysis was reported by Litchfield et al. (3), who described an immunoassay for digoxin in which ouabain-mellitin triggers the release of alkaline phosphatase entrapped in a liposome. Stanley et al. (4) extended substrate cycling by incorporating a color reaction (formation of a formazan dye) directly into a NAD⁺/NADH cycle. They reported a cycling time of about 50 min⁻¹, and used the system in an enzyme immunoassay of thyrotropin.

Here we demonstrate a new concept for chemical amplification, which we call "substrate-leash amplification" (SLA).³ Both catalytic and multiplicative mechanisms are involved. The key feature is that a chain-cutting enzyme or enzyme component is attached to a surface via its substrate "leash". The conditions of the assembly restrict spontaneous release of the enzyme or its components, but when free enzyme is introduced that cleaves the substrate leash, a cascade of released enzyme begins. Our initial SLA system comprises the S-peptide and S-protein fragments of ribonuclease A (EC 3.1.27.5) and a polycytidylic acid (poly C) leash.

Materials and Methods

Materials

Polycytidylic acid (5'), 2',3'-cyclic cytidine monophosphate (cCMP), uridine 5'-triphosphate agarose, bovine pan-

³ Nonstandard abbreviations: SLA, substrate-leash amplification; DAO, 1,8-diaminooctyl; poly C, polycytidylic acid; MI, maleimidobutyl; SPDP, N-succinimidyl-3-(2-pyridyldithio)propionyl; PDP, 3-(2-pyridyldithio)propionyl; GMBS, N-γ-maleimidobutyryloxysuccinimide; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin.

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